AWARD NUMBER: **W81XWH-12-2-0025**

TITLE: **Effect of Antimicrobial Peptide KSL-W on Human Gingival Tissue and *C. albicans* Growth, Transition and Secreted Aspartyl Proteinase (SAPS) 2, 4, 5 and 6 Expressions.**

PRINCIPAL INVESTIGATOR: **Dr Mahmoud Rouabhia, Dental Faculty, University of Laval**

CONTRACTING ORGANIZATION: **University of Laval**

REPORT DATE: **April 201**3

TYPE OF REPORT: **Annual Report**

PREPARED FOR: **U.S. Army Medical Research and Materiel Command**

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;

Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE April 2013	**2. REPORT TYPE** Annual	**3. DATES COVERED** 1 Apr 2012 - 31 Mar 2013

4. TITLE AND SUBTITLE
Effect of Antimicrobial Peptide KSL-W on Human Gingival Tissue and C. albicans Growth, Transition and Secreted Aspartyl Proteinase (SAPS) 2, 4, 5 and 6 Expressions.

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-2-0025

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr Mahmoud Rouabhia

Email: mahmoud.rouabhia@fmd.ulaval.ca

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Laval
QUEBEC, CA G1V 0A6

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The antifungal armamentarium for the treatment of systemic fungal infections has increased in recent years. Although very helpful to control/eliminate fungal infections, the available antifungal drugs do have some limitations such as antifungal drug resistance. As an example, azole resistance is an issue in patients with chronic mucocutaneous candidiasis caused by C. albicans in the context of HIV-infected individuals with recurrent oropharyngeal and esophageal candidiasis. A similar trend in vaginal isolates of C. albicans has been seen in women prone to recurrent vaginal candidiasis exposed to long-term fluconazole (Bulik et al., 2009),(Shahid and Sobel, 2009). In the latter scenario – fortunately relatively rare to date – therapeutic options available for oral management of fluconazole-reduced susceptibility C. albicans are few, resulting in the inconvenient use of long-term topical imidazoles. These facts have generated greater interest in the development of new antifungal drugs using various synthetic and naturally occurring antimicrobial molecules. Natural antimicrobial peptides, such as defensins produced by epithelial cells, showed a broad range of antibacterial activity and could play a role in preventing microbial infections(Decanis et al., 2009),(Zaslof, 2002). These antimicrobial peptides generally exhibit selective toxicity for microorganisms and show fewer propensities to induce microbial resistance.

15. SUBJECT TERMS- nothing listed

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU		**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

1-INTRODUCTION: The antifungal armamentarium for the treatment of systemic fungal infections has increased in recent years. Although very helpful to control/eliminate fungal infections, the available antifungal drugs do have some limitations such as antifungal drug resistance. As an example, azole resistance is an issue in patients with chronic mucocutaneous candidiasis caused by *C. albicans* in the context of HIV-infected individuals with recurrent oropharyngeal and esophageal candidiasis. A similar trend in vaginal isolates of *C. albicans* has been seen in women prone to recurrent vaginal candidiasis exposed to long-term fluconazole (Bulik *et al.*, 2009)'(Shahid and Sobel, 2009). In the latter scenario – fortunately relatively rare to date – therapeutic options available for oral management of fluconazole-reduced susceptibility *C. albicans* are few, resulting in the inconvenient use of long-term topical imidazoles. These facts have generated greater interest in the development of new antifungal drugs using various synthetic and naturally occurring antimicrobial molecules. Natural antimicrobial peptides, such as defensins produced by epithelial cells, showed a broad range of antibacterial activity and could play a role in preventing microbial infections(Decanis *et al.*, 2009)'(Zaslof, 2002). These antimicrobial peptides generally exhibit selective toxicity for microorganisms and show fewer propensities to induce microbial resistance.

Scope of the research **:** For the development of alternative antifungal treatment, we have synthesized an α-helical antimicrobial decapeptide, KSL (KKVVFKVKFK), and its analog, KSL-W (KKVVFWVKFK)(Na *et al.*, 2007), which possess a broad range of antibacterial activity. It killed selected strains of non-oral and oral pathogens, including mutans streptococci. In combination with a surface-active agent, benzalkonium chloride, the peptide significantly reduces *in vitro* biofilm growth(Dixon *et al.*, 2008; Dixon *et al.*, 2009; Leung *et al.*, 2005; Leung *et al.*, 2009).

2-KEYWORDS: Fungal treatment, *C. albicans*, Antifungal molecules, fungi resistance, antimicrobial peptides, cationic peptides, chemical peptides, KSL-W.

3-**ACCOMPLISHMENTS:** There was no change as to the original proposal.

The primary goals of this study were:

1. To investigate the effect of antimicrobial peptide KSL-W (developed by the US Army Dental and Trauma Research Detaachment) on *C. albicans* growth and biofilm formation under the activation of virulence genes.
2. To investigate the effect of KSL-W on human gingival cell growth and migration/wound healing.

Accomplished work

1) **Major activities:** We conducted a complete study evaluation the effect of KSL-W on *C. albicans* growth and pathogenesis.

2) **We specifically** studied the *C. albicans growth, transition and virulence gene (*EFG1, NRG1, EAP1, HWP1, and SAP 2-4-5-6) expression following yeast contact with KSL-W.

3) ***Results:*** We demonstrated that KSL-W markedly reduced *C. albicans* growth at both early and late incubation times. The significant effect of KSL-W on *C. albicans* growth was observed beginning at ten µg/ml after five h of contact by reducing *C. albicans* transition and at 25 µg/ml by completely inhibiting *C. albicans* transition. Cultured *C. albicans*under biofilm-inducing conditions revealed that both KSL-W and amphotericin B significantly decreased biofilm formation at 2, 4, and six days of culture. KSL-W also disrupted mature *C. albicans* biofilms. The effect of KSL-W on *C. albicans* growth, transition, and biofilm formation/disruption may thus occur through gene modulation, as the expression of various genes involved in *C. albicans* growth, transition and biofilm formation were all down-regulated when *C. albicans* was treated with KSL-W. The effect was greater when *C. albicans* was cultured under hyphae-inducing conditions. These data provide new insight into the efficacy of KSL-W against *C. albicans* and its potential use as an antifungal therapy.

4) ***Other achievements.*** We did not perform the studies related to the second objective because the lack of funding. There was no funding transferred from the funding agency to the university to do the study.

All needed information's related to the different protocols we used, and the figures about to the results are included in the published paper (see appendix 1). As a conclusion, we clearly demonstrated the efficacy of KSL-W on influencing *C. albicans* growth, phase transition and expression of virulence genes. This suggested the usefulness of KSL-W against *C. albicans* pathogenesis. However, the use of KSL-W for clinical applications should first be supported by *in vitro* studies using human cells to confirm the non-toxicity of the peptide.

Opportunities for training:

1. A student was involved in the project under his Master degree achievement. He was involved in the experimental protocols with *C. albicans*, data collections, and manuscript preparation.
2. The student contributed in presenting the work on antimicrobial peptide KSL-W on the research day of the Faculty of Dentistry, and at the Medical faculty of Laval University.

Results dissemination:

The results were disseminated through publications and presentations.

Plan for the next reporting period:

If the money transfer occurs to perform the second objective, we will be more than happy to perform the study related to objective 2 investigating the effect of KSL-W on human gingival cell growth and migration/wound healing.

4-IMPACT: The major accomplishment is the understanding the mechanism(s) by which antimicrobial peptide KSL-W in reducing *C. albicans* pathogenesis *in vitro*.

The impact on the development of the principal discipline(s) of the project

We clearly demonstrated that KSL-W was effective in reducing *C. albicans* growth, transition through the down-regulation of certain important genes involved in biofilm formation. This

consolidates the previous studies on inhibition of bacterial growth and suggests the potential use of KSL-W against microbial infections in human.

The impact on other disciplines
Nothing to Report.

The impact on technology transfer
Nothing to Report.

The impact on society beyond science and technology
Eventually the data generated through the first objective may suggest the use of KSL-W to control infection and minimize the emergence of microbial resistance. Such improvement may be of great economic improvement in reducing infection and promoting person health. This will allow more active work, thus economic improvement. It may also be very important for the design of new antimicrobial molecules, thus giving good treatment alternative, and creating more jobs.

5-CHANGES/PROBLEMS:
Nothing to report.

Changes in approach and reasons for change
Nothing to report.

Changes that had a significant impact on expenditures
There was no delay in performing the first objective. Thanks to the University Laval Financial department for providing support in advance to pay for different expenses and the studentship. This allowed us to perform most of the experiments on time which led to collection of useful data and the publication of a peer-reviewed paper. The second objective could not be performed, because there was lack of funds coming from the funding agency to the University Laval.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

Significant changes in use or care of human subjects
Nothing to Report.

Significant changes in use or care of vertebrate animals.
Nothing to Report.

Significant changes in use of biohazards and/or select agents.
Nothing to Report.

6-PRODUCTS:
Nothing to Report.

Publications, conference papers, and presentations

Journal publications.

Theberge S, Semlali A, Alamri A, Leung KP, **Rouabhia M**. C. albicans growth, transition, biofilm formation, and gene expression modulation by antimicrobial decapeptide KSL-W. BMC Microbiol. 2013 Nov 7;13:246. doi: 10.1186/1471-2180-13-246

Status of publication: Published

Acknowledgement of federal support: Yes

Abstracts:

1. Theberge Simon, Jacques Éric and Leung Kai P and Rouabhia Mahmoud. Un nouveau peptide antimicrobien contrôle la virulence de Candida en réduisant sa viabilité via un processus d'apoptose et de nécrose. Journée de la recherche GREB/FMD, le 10 mai, 2013

Status of publication:	Published in the event proceeding
Presentation:	Oral
Acknowledgement of federal support:	Yes

2. Théberge Simon, Jacques Éric, Leung Kai P and **Rouabhia Mahmoud**. Un nouveau peptide antimicrobien contrôle la virulence de Candida en réduisant sa viabilité via un processus d'apoptose et de nécrose. Journée de la recherche faculté de médecine – 30 mai 2013, Université Laval. Québec.

Status of publication:	Published in the event proceeding
Presentation:	Oral
Acknowledgement of federal support:	Yes

3. Théberge Simon, Semlali Abdelhabib, Alamri Abdullah, Leung P. Kai, and **Rouabhia Mahmoud**. Le KSL-W réduit la croissance de Candida albicans et la formation de biofilm en diminuant l'expression de plusieurs gènes de virulence. 81^{e} Congrès de l'Acfas, du 6 au 10 mai 2013, Université Laval, Québec, Canada.

Status of publication:	Published in the event proceeding
Presentation:	Oral
Acknowledgement of federal support:	Yes

Other publications, conference papers, and presentations.

None

Website(s) or other Internet site(s)

None

Technologies or techniques

None

Inventions, patent applications, and/or licenses
None

Other Products
None

7-PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

See below Tables.

Name:	Simon Theberge
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	University Laval Student
Nearest person month worked:	20 h a week
Contribution to Project:	M. Theberge has performed a large part of the experimental protocol related to the evaluation of the effect of KSL-W on C. albicans.
Funding Support:	

Name:	M. Abdelhabib Semlali
Project Role:	Post-Doc
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	Five h a week
Contribution to Project:	M. Semlali has supervised the grad student.
Funding Support:	Laval University Foundation

Name:	Abdullah Alamri
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	University Laval student
Nearest person month worked:	5
Contribution to	M. Alamri contributed, with the grad student M. Teberge to

Project:	perform the genes expression protocols and data collection and analyses.
Funding Support:	

Name:	Leung KP
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	K. Leung has contributed the study design and manuscript revision.
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report.

The Organizations involved as partners

The University Laval as an involved organization.

At the dental Faculty of Laval University, I was able to use different equipment to perform the study and get publishable results. Without such in-kind supports, the study would be very difficult/impossible to realize. The equipments at the research center of the Dental Faculty at Laval University were obtained because of the financial supports of University Laval and different funds that Dr Rouabhia obtained previously from different funding agencies. These include the CIHR, NSERC, FRSQ, the fonds Émile-Beaulieu at the dental Faculty of Laval University, and so.

8-SPECIAL REPORTING REQUIREMENTS

We need a no cost extension to perform what left from the study. The Pi and the University of Laval will send scientific and financial final reports to Ms. Wendy Baker and/or Mr. Robert Jones. We would like to have a 24 months no cost extension because the original agreement ended in 2014. This should give us time to tie all the loose ends of the study to have final reports on time.

9-APPENDICES:

Appendix 1: Published paper
Appendix 2: Presented abstracts (1, 2 and 3).

Theberge et al. BMC Microbiology 2013, 13:246
http://www.biomedcentral.com/1471-2180/13/246

BMC Microbiology

RESEARCH ARTICLE Open Access

C. albicans growth, transition, biofilm formation, and gene expression modulation by antimicrobial decapeptide KSL-W

Simon Theberge[1], Abdelhabib Semlali[1,2], Abdullah Alamri[1], Kai P Leung[3] and Mahmoud Rouabhia[1*]

Abstract

Background: Antimicrobial peptides have been the focus of much research over the last decade because of their effectiveness and broad-spectrum activity against microbial pathogens. These peptides also participate in inflammation and the innate host defense system by modulating the immune function that promotes immune cell adhesion and migration as well as the respiratory burst, which makes them even more attractive as therapeutic agents. This has led to the synthesis of various antimicrobial peptides, including KSL-W (KKVVFWVKFK-NH_2), for potential clinical use. Because this peptide displays antimicrobial activity against bacteria, we sought to determine its antifungal effect on *C. albicans*. Growth, hyphal form, biofilm formation, and degradation were thus examined along with EFG1, NRG1, EAP1, HWP1, and SAP 2-4-5-6 gene expression by quantitative RT-PCR.

Results: This study demonstrates that KSL-W markedly reduced *C. albicans* growth at both early and late incubation times. The significant effect of KSL-W on *C. albicans* growth was observed beginning at 10 μg/ml after 5 h of contact by reducing *C. albicans* transition and at 25 μg/ml by completely inhibiting *C. albicans* transition. Cultured *C. albicans* under biofilm-inducing conditions revealed that both KSL-W and amphotericin B significantly decreased biofilm formation at 2, 4, and 6 days of culture. KSL-W also disrupted mature *C. albicans* biofilms. The effect of KSL-W on *C. albicans* growth, transition, and biofilm formation/disruption may thus occur through gene modulation, as the expression of various genes involved in *C. albicans* growth, transition and biofilm formation were all downregulated when *C. albicans* was treated with KSL-W. The effect was greater when *C. albicans* was cultured under hyphae-inducing conditions.

Conclusions: These data provide new insight into the efficacy of KSL-W against *C. albicans* and its potential use as an antifungal therapy.

Keywords: Antimicrobial peptide, KSL-W, *C. albicans*, Growth, Hyphae, Gene, EFG1, NRG1, HWP1, SAPs

Background

The innate defense system plays a key role in protecting the host against microorganism-fueled infections such as candidiasis caused by *Candida albicans. C. albicans* colonizes several body sites, including the oral cavity; however, as a commensal organism, it causes no apparent damage or inflammation in the surrounding tissue [1,2]. *C. albicans* is a polymorphic organism that adheres to different surfaces in the body and can grow as yeast, pseudohyphae, and hyphae [3], usually in the form of biofilm. *C. albicans* transition, biofilm formation, and pathogenesis are under the control of various genes. The *HWP1* gene encodes the hyphal cell wall protein, which is a hyphal-specific adhesin that is essential to biofilm formation [4]. The involvement of *HWP1* in *C. albicans* adhesion is supported by the *EAP1* gene which encodes a glucan-crosslinked cell wall protein (adhesin Eap1p). Together, these components mediate *C. albicans* adhesion to various surfaces, such as epithelial cells and polystyrene [5]. Like many other genes, *HWP1* and *EAP1* are downstream effectors of EFG1 and NRG1 as transcription factors [6,7]. *EFG1* mutant strain has been shown to exhibit defects in growth, biofilm formation, and virulence [8], while NRG1 represses filamentous growth [3]. This occurs through the DNA binding protein Nrg1p in conjunction with the global transcriptional repressor

* Correspondence: mahmoud.rouabhia@fmd.ulaval.ca
[1]Oral Ecology Research Group, Faculty of Dentistry, Laval University, 2420, rue de la Terrasse, Quebec G1V 0A6, QC, Canada
Full list of author information is available at the end of the article

Tup1p to suppress hyphal formation. Elevated NRG1 expression represses the expression of a number of hypha-specific genes, although NRG1 downregulation is associated with *C. albicans* filaments [3].

C. albicans virulence is also mediated by proteolytic enzymes, including secreted aspartyl proteinases (SAPs) [9,10]. The contribution of SAPs in *C. albicans* adherence, tissue damage, and evasion of host immune responses has been reported [9]. SAP2 is crucial to *C. albicans* growth in protein-containing media [11]. SAP1 and SAP3 are expressed during phenotypic switching [12,13], while SAP4, SAP5, and SAP6 are expressed upon hyphal formation [14], and SAPs 1-6 and 9-10 are involved in the adhesion mechanism to host cells [15].

To control *C. albicans* pathogenesis, the host innate immunity uses small molecules such as proteins and peptides that display a broad antimicrobial spectrum. The number of identified potentially antimicrobial peptides is significant and continues to increase [16]. Antimicrobial peptides often possess common attributes, such as small size, an overall positive charge, and amphipathicity [17,18]; however, they also fall into a number of distinctively diverse groups, including α-helical peptides, β-sheet peptides, peptides with mixed α-helical and β-sheet structures, extended peptides, and peptides enriched in specific amino acids [16].

In humans, epithelial cells and neutrophils are the most important cells producing antimicrobial peptides [19,20]. These peptides are most often antibacterial, although antifungal activity has also been reported [16,21]. The major peptide groups known to date are the histatins, cathelicidins, defensins, and lactoferricins [22]. The antimicrobial activity of these peptides has been reported by different *in vitro* and *in vivo* studies [19,20,22]. Their complex role as well as their contribution to host defenses may be related to the functional interrelationship between innate and adaptive immunity [23,24].

The interest in antimicrobial peptides lies in the possible resistance of microorganisms to conventional antimicrobial strategies used against microbial pathogens in both agriculture and medicine [25,26]. Natural antimicrobial peptides are necessary in the control of microbial infections. For example, the use of AMPs provided protection against such microbial pathogens as fungal pathogens, with no reported effect on the host [27,28]. Based on these promising data, a number of synthetic AMPs have been designed to overcome microbial infections [29]. In the pursuit of a novel alternative antifungal treatment, we developed a synthetic α-helical antimicrobial decapeptide, KSL (KKVVFKVKFK), and its analogue KSL-W (KKVVFWVKFK) [30].

The efficacy of KSL on a wide range of microorganisms has been established [31-33], as well as its ability to disrupt oral biofilm growth [34]. KSL-W, a recently synthesized KSL analogue, was shown to display improved stability in simulated oral and gastric conditions with *in vitro* preserved antimicrobial activity [30]. Furthermore, combined with sub-inhibitory concentrations of benzalkonium chloride, a known cationic surface-active agent [35], KSL was shown to significantly promote bacterial biofilm susceptibility. We also recently demonstrated that KSL-W had a selective effect on *C. albicans* growth, while exhibiting no toxic effect on epithelial cells [36].

As this KSL-W analogue displays a wide range of microbicidal activities, effectively kills bacteria, controls biofilm formation, and destroys intact biofilms, we hypothesized that KSL-W may also possess antifungal potential. Our goal was thus to investigate the ability of KSL-W to inhibit *C. albicans* growth and transition from blastospore to hyphal form. The action of KSL-W on biofilm formation/disruption was also assessed. Finally, we examined the effect of KSL-W on various *C. albicans* genes involved in its growth, transition, and virulence.

Results

Antimicrobial peptide KSL-W reduced *C. albicans* growth and transition from blastospore to hyphal form

C. albicans cultures were incubated with KSL-W for 5, 10, and 15 h to determine whether this antimicrobial peptide had any adverse effect on *C. albicans* growth. As shown in Figure 1, KSL-W significantly reduced *C. albicans* proliferation. After 5 h of contact with KSL-W, the growth inhibition of *C. albicans* was between 30 and 80%, depending on the concentration of KSL-W used (Figure 1A). After 10 h of contact with KSL-W, growth inhibition was significant, beginning at 25 μg/ml (Figure 1B). At later culture periods, *C. albicans* growth continued to be significantly affected by the presence of KSL-W (Figure 1C). Indeed, with 25 μg/ml of KSL-W, *C. albicans* growth was almost half that in the controls (non-treated *C. albicans* cultures), and with 100 μg/ml of KSL-W, *C. albicans* growth was reduced by almost 60%. It is interesting to note that KSL-W in as low as 25 μg/ml was effective at both the early and late culture periods.

As KSL-W contributed to *C. albicans* growth inhibition, we hypothesized that it would also downregulate *C. albicans* transition from yeast form to hyphal phenotype. Yeast cultures supplemented with 10% FBS and the KSL-W peptide were maintained for various incubation periods. As shown in Figure 2, germ tube formation was inhibited as early as 4 h following exposure to the peptide, compared to that in the cultures incubated in the absence of KSL-W. Of interest is the elevated number of *C. albicans* hyphal forms in the negative control culture (no KSL-W or amphotericin B) compared to the low number in the presence of KSL-W. The effect of this

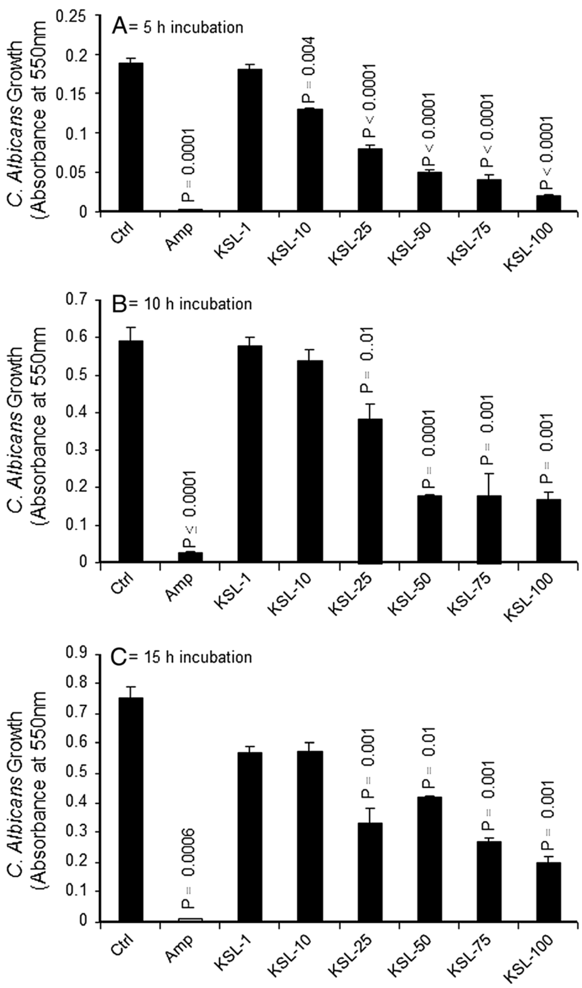

Figure 1 KSL-W inhibited *C. albicans* growth. The yeast was cultured in Sabouraud supplemented medium with or without KSL-W at various concentrations. The cultures were maintained for 5, 10, and 15 h at 37°C, after which time an MTT assay was performed for each culture condition. The growth was plotted as means ± SD of the absorbance at 550 nm. **(A)** *C. albicans* growth with KSL-W for 5 h; **(B)** *C. albicans* growth with KSL-W for 10 h; and **(C)** *C. albicans* growth with KSL-W for 15 h. The levels of significance for *C. albicans* growth in the presence or not of KSL-W or amphotericin B (10 μg/ml) were considered significant at $P < 0 \cdot 05$.

antimicrobial peptide on *C. albicans* transition was also dose-dependent: at 1 μg/ml, a significant number of hyphal forms remained, and at only 5 μg/ml of KSL-W, *C. albicans* transition was completely inhibited (Figure 2). Semi-quantitative analyses using inverted microscope observations to estimate the hyphal forms confirmed the inhibited *C. albicans* transition when treated with KSL-W (Table 1). The density of the hyphae was reduced as early as 4 h of contact with 5 μg/ml of KSL-W. This effect was further supported when *C. albicans* was placed in contact with KSL-W for 8 h (Table 1), thus confirming that KSL-W downregulated *C. albicans* growth and transition.

KSL-W reduced *C. albicans* biofilm formation

As KSL-W contributed to reducing *C. albicans* growth and transition, we sought to determine whether it also displayed inhibitory activity against *C. albicans* biofilm formation. Using a biofilm-promoting scaffold, SEM analyses, and an XTT assay, we were able to demonstrate the inhibitory effect of KSL-W on biofilm formation (Figure 3). SEM analyses revealed a significant density of *C. albicans* in the untreated culture, compared to a lower density in the scaffold in the presence of KSL-W (1 and 25 μg/ml) after 4 days of culture. The decreases obtained with the KSL-W, particularly at 25 μg/ml (Figure 3), were comparable to that obtained with amphotericin B at 10 μg/ml. To confirm these observations, we performed quantitative analyses using the XTT assay. Figure 4A shows that after 2 days of culture, KSL-W was able to inhibit biofilm formation. This inhibitory effect was observed beginning at 25 μg/ml of KSL-W. At concentrations of 50, 75, and 100 μg/ml of KSL-W, the inhibition of *C. albicans* biofilm formation was comparable to that caused by amphotericin B at 10 μg/ml. Similar results were obtained after 4 days (Figure 4B) and 6 days (Figure 4C) of culture for biofilm formation with a persistent inhibitory effect of KSL-W on *C. albicans* biofilm formation.

KSL-W disrupted mature *C. albicans* biofilms

After 6 days of incubation in glucose-rich Sabouraud medium, scaffolds seeded with *C. albicans* strain SC5314 produced mature biofilms displaying highly dense populations of *Candida* cells (Figure 5). Significant reductions and disruptions of the pre-formed *Candida* biofilms were observed when the reference antifungal agent (amphotericin B, 10 μg/ml) was added to the mature biofilms upon further incubation up to 6 days. Similarly, antimicrobial peptide KSL-W at 75 and 100 μg/ml also reduced *C. albicans* density in the biofilms. The observed reduction was noticed with KSL-W concentrations ranging from 25 to 100 μg/ml. Indeed, when quantitatively investigated by XTT reduction assay, the KSL-W-treated biofilms rendered a significantly lower number of cells, as reflected by the lower absorbance readings, than did the untreated control. This effect was observed after 2, 4, and 6 days of treatment with amphotericin B. Furthermore, the effect of KSL-W on the mature *C. albicans* biofilm was comparable to that obtained with amphotericin B (Figure 6).

KSL-W modulated the expression of various *C. albicans* genes

Based on the data showing that KSL-W reduced *C. albicans* proliferation, transition, and biofilm formation, we sought to determine the involvement, if any, of gene regulation. For this purpose, we first investigated the

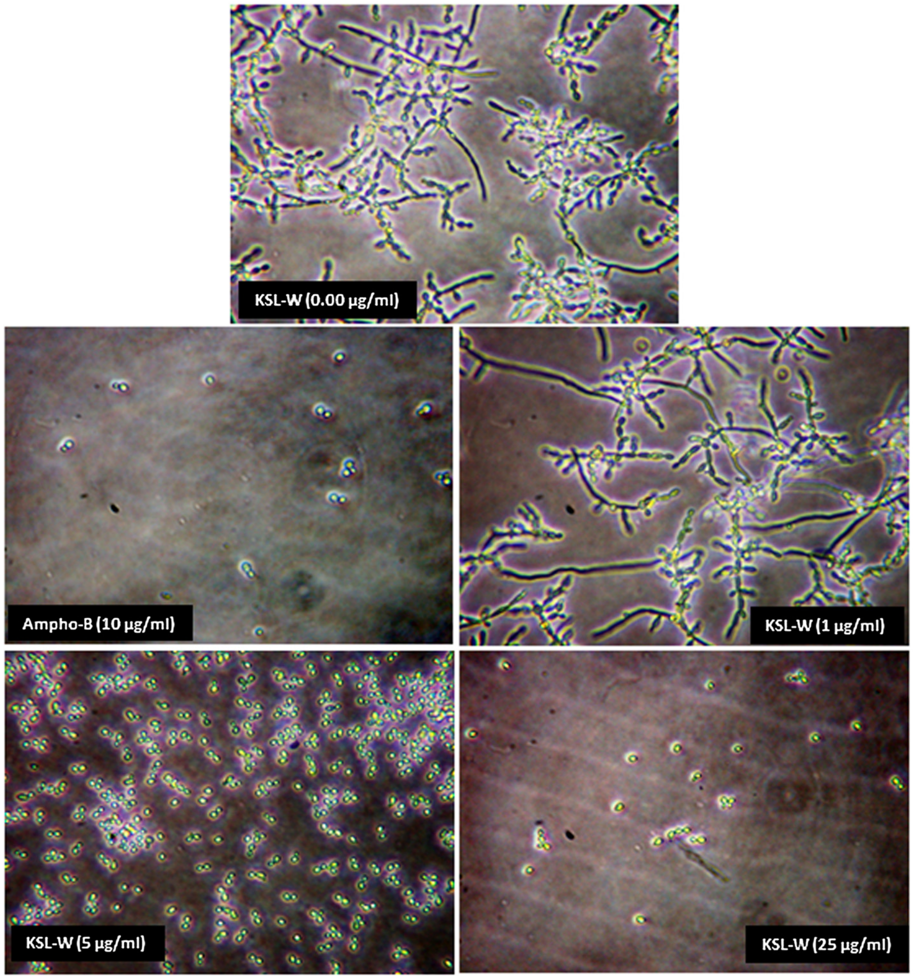

Figure 2 KSL-W inhibited *C. albicans* yeast-to-hyphae transition. *C. albicans* was cultured in Sabouraud medium containing 10% fetal bovine serum with or without KSL-W at various concentrations and was maintained for 4 and 8 h at 37°C. After each time point, the cultures were observed under an inverted microscope and photographed. Representative photos of the morphological changes after 4 h of culture are presented.

Table 1 Estimation of hyphae forms in the *C. albicans* culture

Active molecules	Concentration (µg/mL)	Transition at 4 h	Transition at 8 h
Negative control	0	++	++
KSL-W	1	++	++
	5	-	-
	10	-	-
	15	-	-
	25	-	-
	100	-	-
Amphotericin B	1	-	-

This Table depicts the presence of hyphae following 4 and 8 h treatments of *C. albicans* with and without KSL-W or amphotericin B. (–) refers to the absence hyphae form, and (++) refers to the presence high number of hyphae forms. These data were estimated after evaluation over 20 fields from each culture condition, by two independent and blinded examiners.

effect of KSL-W on the activation/repression of various *C. albicans* genes when cultured under normal non-hyphae-inducing conditions. The data in Table 2 indicate that the HWP1 gene was significantly downregulated following exposure of the *C. albicans* to KSL-W for 6 h. This downregulation was comparable to that observed in the amphotericin B treatment. Similarly, SAPs 2, 4, 5, and 6 were significantly downregulated by KSL-W treatment after 6 h (Table 2). This effect was observed with both low and high concentrations of KSL-W. Furthermore, the EAP1 gene, which encodes a glycosylphosphatidylinositol-anchored, glucan-crosslinked cell wall protein in both adhesion and biofilm formation *in vitro* and *in vivo*, was also affected by the KSL-W treatment. Moreover, the expression of this gene was downregulated by KSL-W, yet was upregulated (up to 5-fold) by amphotericin B.

Two other genes involved in regulating *C. albicans* morphogenesis, namely, *EFG1* and *NRG1*, are known to

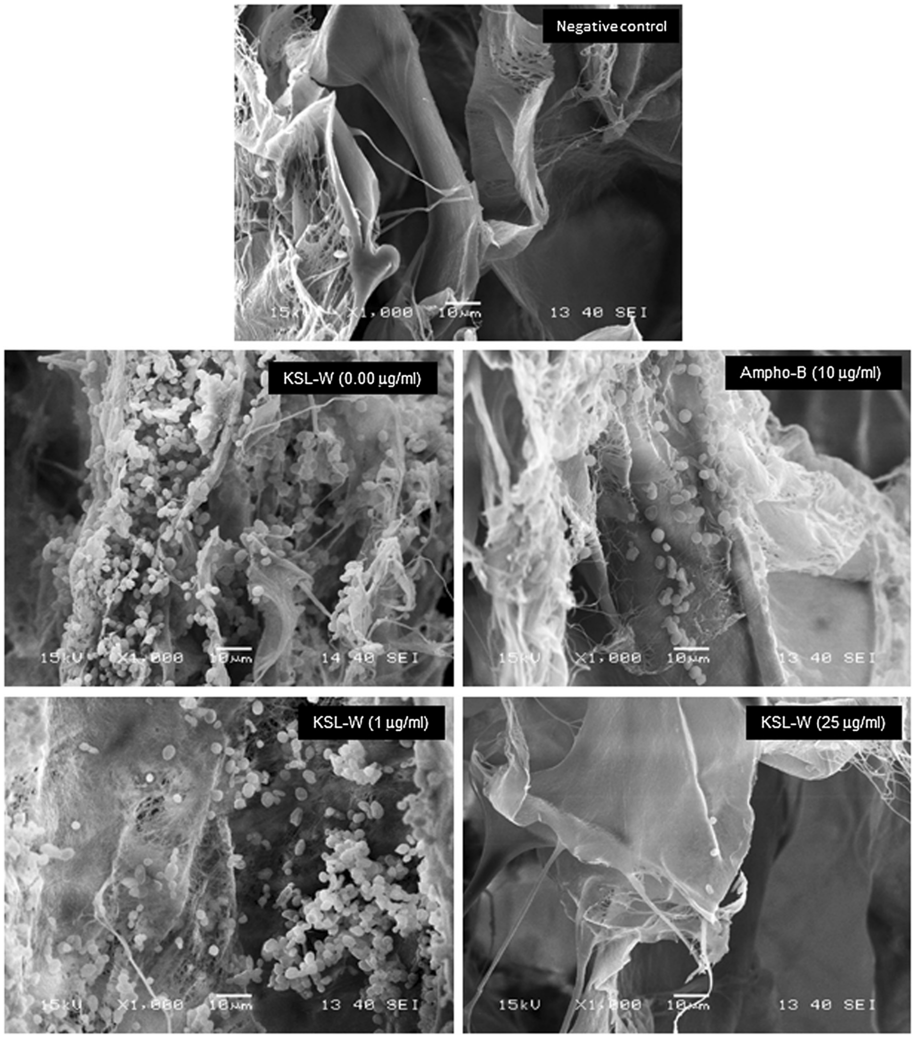

Figure 3 Scanning electron microscope analyses of the biofilm formation. *C. albicans* was cultured in Sabouraud medium with or without KSL-W at various concentrations for 4 days in a porous 3D collagen scaffold. Cultures in the presence of amphotericin B (10 µg/ml) were used as the positive controls. Following incubation, the samples were prepared as described in the Methods section and were observed under a scanning electron microscope. Negative control refers to the non-seeded scaffolds.

be hyphae repressors. In our study, amphotericin B increased both EFG1 and NRG1 mRNA expression, with twice as much expression for *NRG1* than for *EFG1* (Table 3), while KSL-W induced a less significant increase of *EFG1* and *NRG1* mRNA expression. Of interest is that a low KSL-W concentration (25 µg/ml) induced greater gene expression (Table 3).

In a second set of experiments, *C. albicans* was cultured under hyphae-inducing conditions (fetal calf serum-enriched medium with incubation at 37°C) in the presence or not of KSL-W, after which time gene expression/repression was investigated. The data in Table 4 reveal that similar to the results obtained with amphotericin-B, the *HWP1* gene was significantly ($p < 0.0001$) downregulated when *C. albicans* was exposed to KSL-W for 3 h, confirming the results obtained under non-hyphae growth conditions.

SAP genes were also modulated by KSL-W treatment. Table 4 shows that after 3 h of exposure, SAPs 2, 4, 5, and 6 were significantly ($p < 0.05$) downregulated by the KSL-W treatment. In contrast, with amphotericin-B, a significant ($p < 0.05$) increase of *SAP*s 2, 4, and 6 and a decrease of *SAP*5 was observed. It is interesting to note the opposite modulatory effects of KSL-W and amphotericin-B on *SAP* gene expression. After 6 h of treatment with KSL-W, a significant decrease of each tested *SAP* gene was observed in the exposed *C. albicans*, whereas after treatment with amphotericin-B, these same *SAP* genes increased, thus confirming the antagonistic behavior of KSL-W and amphotericin-B on *SAP* gene expression.

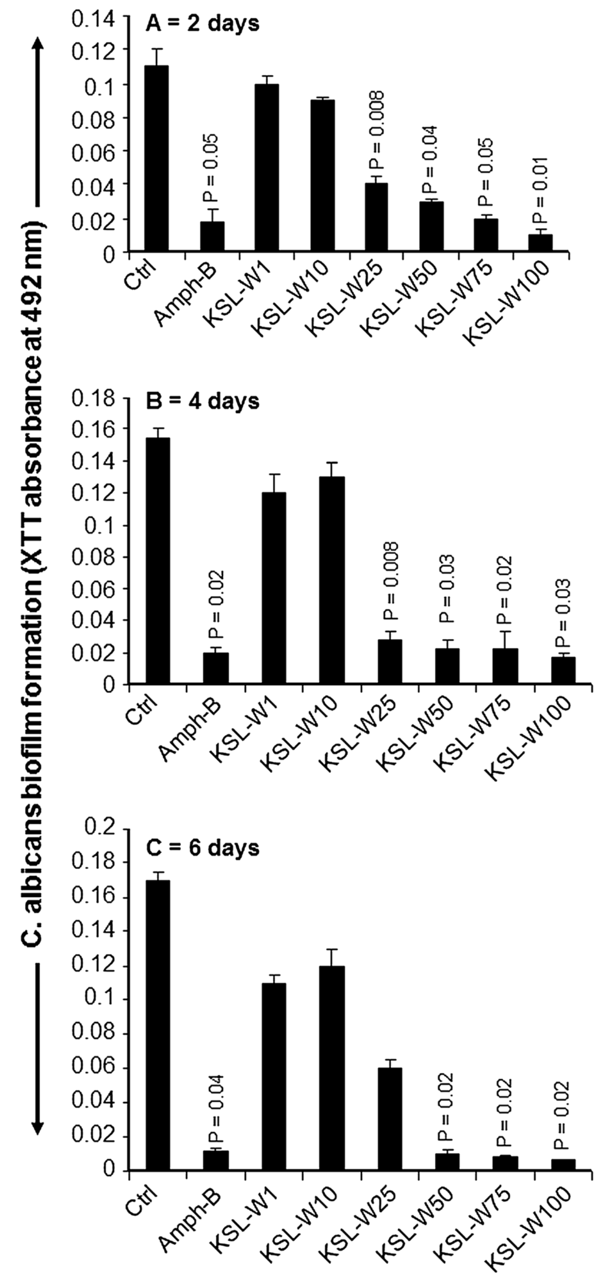

Figure 4 Quantitative measurement of the reduced biofilm formation with KSL-W. *C. albicans* was cultured on a 3D porous scaffold in the presence of KSL-W for 2, 4, and 6 days. After each culture period, the samples were supplemented with XTT solution and incubated for 5 h at 37°C. The absorbance at 450 nm was measured to quantify XTT metabolic product intensity proportional to the number of viable cells. **(A)** 2 days; **(B)** 4 days; **(C)** 6 days. Results are means ± SD for three different separate experiments.

C. albicans EAP1 gene expression was unchanged after 3 h with KSL-W, but significantly ($p < 0.001$) decreased after 6 h, while the expression of this gene was upregulated (close to six folds) by amphotericin B (Tables 4 and 5). Amphotericin B increased NRG1 mRNA expression almost threefold, with no significant effect on the *EFG1* gene, yet significantly decreased HWP1 gene expression. On the other hand, after 3 h (Table 4) and 6 h (Table 5) of incubation, KSL-W downregulated EFG1, NRG1, and HWP1 mRNA expression. Of interest is that except for similar downregulatory effects on *HWP1* gene expression, KSL-W and amphotericin-B produced once again opposite results regarding *EFG1*and *NRG1* gene expression.

Discussion and conclusions

We demonstrated that KSL-W was effective in inhibiting *C. albicans* growth at short and long culture periods. Although growth inhibition obtained with KSL-W was less than that obtained with amphotericin B, the effects of KSL-W nevertheless remain significant ($p < 0.01$). The growth inhibition effects of KSL-W are in accordance with previously reported findings [37] showing a downregulation of *C. albicans* activity induced by a bacteriocin-like peptide isolated from *Lactobacillus pentosus*. Furthermore, our results support other findings [38] reporting the effectiveness of KSL-W in disrupting *P. gingivalis*-induced hemagglutination and its synergistic interaction with host AMPs engaged in innate defense. The results strongly suggest that KSL-W is also effective against fungal growth and may be suitable for use to control *C. albicans* infections. Further studies on the possible synergistic effect of amphotericin B and KSL-W against *C. albicans* growth may provide insight.

C. albicans pathogenesis can also take place through the transition from blastospore to hyphal form [39,40]. Our results indeed show that KSL-W completely inhibited *C. albicans* transition with a concentration as low as 5 μg/ml. These data are consistent with those of other studies with naturally occurring antimicrobial peptides (e.g., β-defensins) which were effective in blocking the morphological shift of *Candida* from yeast to hyphae [41,42]. Thus KSL-W may possibly contribute to the control of *C. albicans* infection by reducing cell growth and yeast-hyphae transition. The effect of KSL-W on *C. albicans* growth can occur either through cytolysis or cell membrane disruption, resulting in cell death similar to what has been demonstrated with histatin-5 [43,44]. Indeed, it was shown that histatin-5 induces the selective leakage of intracellular ions and ATP from yeast cells. This is caused by the translocation of histatin-5 into the intracellular compartment and accumulates to a critical concentration [45]. Further studies are thus warranted to shed light on the fungicidal mechanism of KSL-W.

C. albicans growth and transition from blastospore to hyphal form are particularly important for biofilm formation and *C. albicans* virulence because a strain that is genetically manipulated to grow exclusively in the yeast form is greatly hindered in generating biofilms. In addition, a variety of *C. albicans* mutants known to be

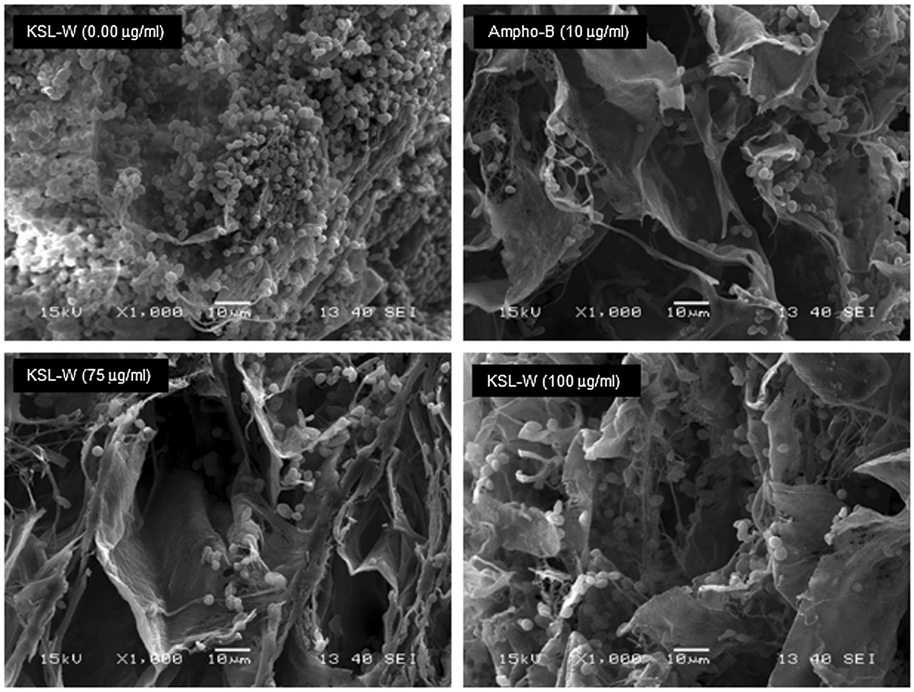

Figure 5 Biofilm ultrastructure following KSL-W treatment. *C. albicans* was cultured in Sabouraud medium without KSL-W for 6 days to promote biofilm formation and maturation. The resulting biofilms were then treated or not with KSL-W or amphotericin B for 6 days, with medium and peptide refreshing every 2 days. Following incubation, the samples were prepared as described in the Methods section and observed under a scanning electron microscope.

unable to form hyphae also show biofilm defects [46,47]. As KSL-W significantly reduced *C. albicans* growth and inhibited its transition from yeast to hyphae, this suggests that KSL-W may inhibit *C. albicans* biofilm formation. Our findings indicate that KSL-W was indeed able to reduce biofilm formation and that its effect was comparable to that obtained with amphotericin B, a well-known antifungal molecule. Also of interest is that a significant inhibition of *C. albicans* biofilm formation was obtained at a concentration of as low as 25 µg/ml of KSL-W antimicrobial peptide. These useful data are comparable to those of other studies showing the positive action of synthetic peptide in controlling and preventing microbial biofilm formation [48]. Thus, with its significant impact in reducing *C. albicans* biofilm formation, KSL-W may show potential for several novel applications in the clinical setting. Further investigations will elucidate this effect.

Biofilm formation can be controlled with anti-biofilm molecules prior to its development, although this is not actually the case in clinical applications, as antifungal and microbial molecules cannot be used on a daily basis to prevent biofilm formation. An effective molecule should ideally be able to prevent biofilm formation, but more importantly to disrupt biofilms that are already formed. We therefore questioned whether KSL-W was capable of disrupting mature *C. albicans* biofilm.

We proceeded to examine the impact of KSL-W on mature biofilm formation and demonstrated a significant disruption of these biofilms following contact with KSL-W, thus suggesting the possible use of this antimicrobial peptide to reduce/eliminate mature biofilms. Further studies should confirm such observations and demonstrate how KSL-W reduces or disrupts *C. albicans* biofilms.

Once it reaches the cell, KSL-W can potentially act on the cytoplasmic membrane as well as on intracellular targets [49-51]. The action of KSL-W against *C. albicans* may operate through the modulated expression of certain *C. albicans* genes that control growth [52], transition [53], and biofilm formation [54]. We therefore examined the effect of KSL-W on a number of genes either directly or indirectly involved in phase transition and biofilm formation. *EFG1* and *NRG1* expression was assessed under hyphae/non-hyphae-inducing conditions. Our results show that KSL-W increased NRG1 mRNA expression twofold under non-hyphae-inducing conditions; however, under hyphae-inducing conditions, KSL-W significantly reduced *NRG1* gene expression. These findings contrast with other reports that an increased NRG1 expression contributes to repressing various hypha-specific genes [55,56]. This confirms that the effect of KSL-W in controlling *C. albicans* virulence does not take place through NRG1. KSL-W was also able to

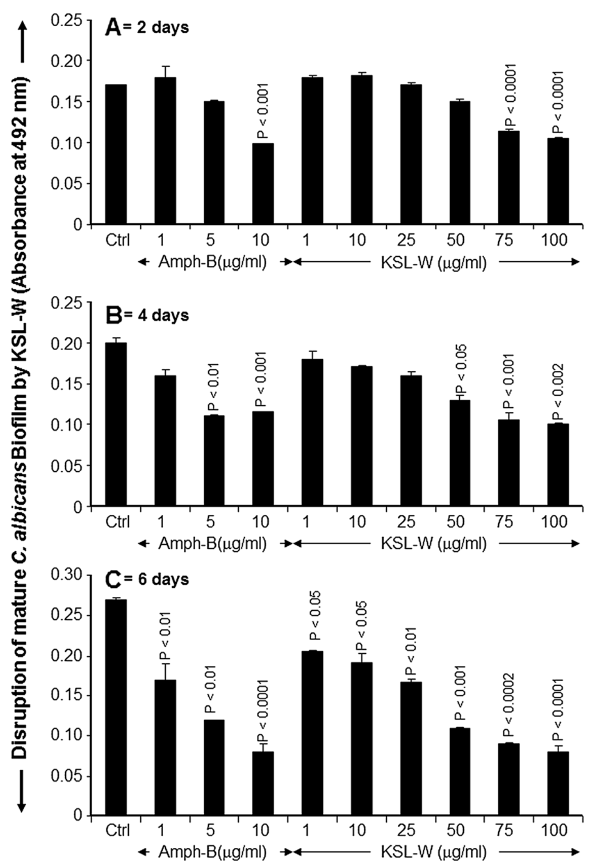

Figure 6 Decrease of biofilm mass following KSL-W treatment. *C. albicans* was cultured in a 3D porous scaffold in Sabouraud medium for 6 days to promote biofilm formation and maturation. The resulting biofilms were exposed or not to KSL-W or amphotericin B for 2, 4, and 6 days. Medium and peptide were refreshed every 2 days. Following each treatment period, the samples were supplemented with XTT solution and subsequently incubated for 5 h at 37°C. Absorbance at 450 nm was measured to quantify XTT metabolic product intensity proportional to the number of viable cells. **(A)** 2 days; **(B)** 4 days; **(C)** 6 days. Results are means ± SD for three separate experiments.

decrease EFG1 mRNA expression, when *C. albicans* was maintained under hyphae-inducing conditions.

EFG1p has been found to be a central regulator of *C. albicans*, as it is required for the development of a true hyphal growth form, and EFG1 is considered to be essential in the interactions between *C. albicans* and human host cells [7,8]. The downregulation of this gene by KSL-W points to the singular role of this antifungal peptide. Thus the effect of KSL-W on *C. albicans* transition can be manifested through a repression of certain genes, such as *EFG1* and *NRG1*.

KSL-W has a significant inhibitory effect on EAP1 mRNA expression. As a member of the GPI-CWP family [5,57], deleting EAP1 can reduce the adhesion of *C. albicans* to different surfaces. This suggests that treatment with KSL-W may reduce EAP1 expression, which in turn may contribute to reducing *C. albicans* adhesion and ultimately, biofilm formation and pathogenesis. KSL-W was also shown to reduce HWP1 mRNA expression, particularly when *C. albicans* was cultured under hyphae-inducing conditions.

HWP1 is a downstream component of the cAMP-dependent PKA pathway and is positively regulated by EFG1 [58]. The transcript level of HWP1 decreased with the KSL-W treatment at low and high concentrations. These data suggest that KSL-W indeed impacts the activity of the cAMP–EFG1 pathway and leads to an alteration of *C. albicans* growth and morphogenesis. Further studies are therefore required to investigate the invasion/virulence of KSL-W-treated *C. albicans*.

It is well known that *Candida* pathogenesis can be established by virtue of *Candida* growth and yeast-to-hyphae morphogenesis. Specific *SAP* genes were found to be preferentially expressed by *Candida* hyphal forms [10,15,59]. Because KSL-W downregulated *C. albicans* growth and transition, this may have occurred through a modulation of the *SAP* genes. Our findings confirm that KSL-W is capable of decreasing SAP2, SAP4, SAP5, and SAP6 mRNA expression in *C. albicans* which may lead to reducing *C. albicans* virulence [60-62].

Our study thus establishes, for the first time, a clear link between an antimicrobial peptide (KSL-W), hyphae

Table 2 Gene expression (6 h) under non-hyphae inducing culture conditions

Gene	Untreated *C. albicans*	Amphotericin B		KSL-W 25 µg/ml		KSL-W 100 µg/ml	
	Fold change[1]	Fold change[1]	p-value[2]	Fold change[1]	p-value[2]	Fold change[1]	p-value[2]
SAP2	0.99	0.57	0.001	0.24	<0.001	0.11	<0.001
SAP4	0.96	0.19	<0.001	0.29	<0.001	0.14	<0.001
SAP5	1.00	0.08	<0.001	0.16	<0.001	0.06	<0.001
SAP6	1.00	0.05	<0.001	0.14	<0.001	0.04	<0.001
EAP1	1.00	4.91	0.028	0.4	<0.001	0.29	<0.001
HWP1	1.00	0.01	<0.001	0.6	0.032	0.02	<0.001

[1]Fold change was calculated by PCR product of the gene of interest/the PCR product of ACT1 (the house keeping gene), and normalized to the negative control of untreated *C. albicans* where the expression was considered equal to 1.
[2]P-values were obtained after comparison of test to negative control (untreated *C. albicans*).

Table 3 Gene expression (3 h) under non-hyphae inducing culture conditions

Gene	Untreated *C. albicans*	Amphotericin B		KSL-W 25 μg/ml		KSL-W 100 μg/ml	
	Fold change[1]	Fold change[1]	p-value[2]	Fold change[1]	p-value[2]	Fold change[1]	p-value[2]
EFG1	1.00	5.71	<0.001	2.76	<0.001	1.98	0.073
NRG1	1.00	10.99	<0.001	1.77	<0.001	1.4	0.086

[1]Fold change was calculated by PCR product of the gene of interest/the PCR product of ACT1 (the house keeping gene), and normalized to the negative control of untreated *C. albicans* where the expression was considered equal to 1.
[2]P-values were obtained after comparison of test to negative control (untreated *C. albicans*).

morphogenesis, and hyphae-modulating SAPs 2, 4, 5, and 6. However, the precise interactions between these SAPs and KSL-W during *C. albicans* pathogenesis remain unclear. Additional studies should focus on identifying the role of SAP subfamilies involved in *Candida* invasion as well as the role of KSL-W in controlling *Candida* virulence/pathogenesis in conjunction with host defenses. In conclusion, this study is the first to demonstrate that synthetic antimicrobial peptide KSL-W downregulates *C. albicans* growth and transition, resulting in a decrease in biofilm formation and a disruption of mature biofilm. Also of interest is that these effects may occur through the modulation of *C. albicans* genes *EFG1*, *NRG1*, *EAP1*, *HWP1*, and *SAP*s. Overall results clearly suggest the potential of KSL-W as an antifungal molecule.

Methods

C. albicans

C. albicans strain ATCC-SC5314 was cultured for 24 h on Sabouraud dextrose agar plates (Becton Dickinson, Oakville, ON, Canada) at 30°C. For the *C. albicans* suspensions, one colony was used to inoculate 10 ml of Sabouraud liquid medium supplemented with 0.1% glucose at pH 5.6. The cultures were grown overnight in a shaking water bath for 18 h at 30°C. The yeast cells were then collected, washed with phosphate-buffered saline (PBS), counted with a haemocytometer, and adjusted to 10^7/ml prior to use.

Antimicrobial peptides

KSL-W (KKVVFWVKFK-NH2) was synthesized by standard solid-phase procedures [63] with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry in an automatic peptide synthesizer (model 90, Advanced ChemTech, Louisville, KY, USA). The synthetic peptides were then purified by reverse-phase HPLC (series 1100, Hewlett Packard) by means of a Vydac C18 column. Peptide purity was confirmed by MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) MS (AnaSpec Fremont, CA, USA). The final product was stored in lyophilized format -20°C until use. KSL-W solution was prepared, filtered (0.22 um pore size), and used for the experiments. Amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in distilled water to obtain a 250 μg/ml concentration which was also filtered, with the sterile solution stored at -80°C until use.

Effect of KSL-W on *C. albicans* proliferation

Proliferation was investigated by placing 10^4 *C. albicans* in 200 μL of Sabouraud dextrose broth in a round-bottom 96-well plate. The *C. albicans* cultures were supplemented with KSL-W at concentrations of 1, 10, 25, 50, 75, and 100 μg/ml. The negative controls were *C. albicans* cultures not supplemented with KSL-W, while the positive controls were *C. albicans* cultures supplemented with amphotericin B at concentrations of 1, 5, and 10 μg/ml. The plates were incubated for 5, 10, and

Table 4 Gene expression (3 h) under hyphae inducing culture conditions (medium supplemented with 10% fetal calf serum, with culture incubation at 37°C)

Gene	Untreated *C. albicans*	Amphotericin B		KSL-W 25 μg/ml		KSL-W 100 μg/ml	
	Fold change[1]	Fold change[1]	p-value[2]	Fold change[1]	p-value[2]	Fold change[1]	p-value[2]
SAP2	0.99	3.36	0.003	0.78	0.02	0.62	0.003
SAP4	0.96	2.41	0.02	0.44	0.0002	0.24	< 0.0001
SAP5	1.00	0.49	0.0007	0.83	0.03	0.01	< 0.0001
SAP6	1.00	2.56	0.01	0.30	< 0.0001	0.11	< 0.0001
EAP1	1.00	6.06	< 0.001	1.06	0.4	0.99	0.8
EFG1	1.00	1.09	0.6	0.55	0.0004	0.66	0.02
NRG1	1.00	2.45	0.01	0.66	0.0006	0.64	0.0005
HWP1	1.00	0.0055	< 0.001	0.078	< 0.0001	0.0035	< 0.0001

[1]Fold change was calculated by PCR product of the gene of interest/the PCR product of ACT1 (the house keeping gene), and normalized to the negative control of untreated *C. albicans* where the expression was considered equal to 1.
[2]P-values were obtained after comparison of test to negative control (untreated *C. albicans*).

Table 5 Gene expression (6 h) under hyphae inducing culture conditions (medium supplemented with 10% fetal calf serum, with culture incubation at 37°C)

Gene	Untreated *C. albicans*	Amphotericin B		KSL-W 25 μg/ml		KSL-W 100 μg/ml	
	Fold change[1]	Fold change[1]	p-value[2]	Fold change[1]	p-value[2]	Fold change[1]	p-value[2]
SAP2	0.99	8.17	0.009	0.7	0.2	1.31	0.02
SAP4	0.96	2.58	0.03	0.73	0.04	0.72	0.04
SAP5	1.00	0.72	0.007	0.83	0.0004	0.56	0.006
SAP6	1.00	4.01	0.02	0.58	0.01	0.68	0.04
EAP1	1.00	6.36	0.001	0.44	0.008	0.73	0.003
EFG1	1.00	1.78	0.048	0.31	< 0.0001	0.47	0.01
NRG1	1.00	3.97	0.0005	0.37	0.001	0.37	0.05
HWP1	1.00	0.008	< 0.001	0.09	0.001	0.03	< 0.0001

[1]Fold change was calculated by PCR product of the gene of interest/the PCR product of ACT1 (the house keeping gene), and normalized to the negative control of untreated *C. albicans* where the expression was considered equal to 1.
[2]P-values were obtained after comparison of test to negative control (untreated *C. albicans*).

15 h prior to cell growth analyses. *C. albicans* growth was assessed using the (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) MTT assay (Sigma-Aldrich) which measures cell growth as a function of mitochondrial activity [64]. Briefly, an MTT stock solution (5 mg/ml) was prepared in PBS and added to each culture at a final concentration of 10% (v/v). The *C. albicans* cultures were then incubated with the MTT solution at 30°C for 4 h, after which time the plate was centrifuged for 10 min at 1200 rpm and the supernatant was removed. The remaining pellet from each well was then washed with warm PBS, with 200 μl of 0.04 N HCl in isopropanol added to each well, followed by another incubation for 15 min. Absorbance (optical density, OD) was subsequently measured at 550 nm by means of an xMark microplate spectrophotometer (Bio-Rad, Mississauga, ON, Canada). Results are reported as means ± SD of three separate experiments.

Effect of KSL-W on *C. albicans* transition from blastospore to hyphal form

To determine the effect of KSL-W on the yeast-to-hyphae transition, *C. albicans* (10^5 cells) was first grown in 500 ml of Sabouraud dextrose broth supplemented with 0.1% glucose and 10% fetal bovine serum (FBS). KSL-W was then added (or not) to the culture at various concentrations (1, 5, 10, 15, and 25 μg/ml). The negative controls were the *C. albicans* cultures without antimicrobial peptide, while the positive controls represented the *C. albicans* cultures supplemented with amphotericin B (1, 5, and 10 μg/ml). The hyphae-inducing conditions were previously reported [65], consisting of culture medium supplementation with 10% fetal calf serum and subsequent incubation at 37°C. These conditions were used in our experiments. Following incubation for 4 or 8 h, the cultures were observed microscopically and photographed to record *C. albicans* morphology (n = 5) and the density of *C. albicans* transition was measured.

Effect of KSL-W on *C. albicans* gene activation/repression

C. albicans was subcultured overnight in Sabouraud liquid medium supplemented with 0.1% glucose, pH 5.6, in a shaking water bath for 18 h at 30°C. The yeast cells were then collected, washed with PBS, and counted with a hemocytometer, after which time they were co-cultured with or without the antimicrobial peptide under hyphae- or non-hyphae-inducing conditions, as follows.

Effect of KSL-W on gene activation when C. albicans was cultured under non-hyphae-inducing conditions

C. albicans was co-cultured with either KSL-W (1, 25, 100 μg/ml) or amphotericin B (1 μg/ml) or with none of these molecules (controls) in Sabouraud liquid medium supplemented with 0.1% glucose, pH 5.6. The cultures were maintained at 30°C for 3 and 6 h.

Effect of KSL-W on gene activation when C. albicans were cultured under hyphae-inducing conditions

C. albicans was co-cultured with either KSL-W (1, 25, 100 μg/ml) or amphotericin B (1 μg/ml) or with none of these molecules (controls) in Sabouraud liquid medium supplemented with 0.1% glucose, pH 5.6. As previously reported, to promote the transition of *C. albicans* from blastospore to hyphal form, the culture medium was supplemented with 10% fetal calf serum and the incubation was performed for 3 and 6 h at 37°C. Following each culture period under both conditions [68], the cultures were centrifuged 10 min at 13,000 rpm, the supernatants were discarded, and each pellet was suspended thereafter in 0.6 ml of lysis buffer (Glycerol 1 M, EDTA 0.1 M). Glass beads (0.425-0.6 mm in diameter; 0.2 ml) were added to each suspended pellet prior to sonication (4 × 1 min, followed by 2 min of incubation in ice) with

a MiniBead-beater (Biospec Products, Bartlesville, OK, USA). Following cell lysis, the total RNA was extracted from each sample by means of the Illustra RNAspin Mini kit (GE Health Care UK Limited, Buckingham, UK). Concentration, purity, and quality of the isolated RNA were determined using the Experion system and RNA StdSens analysis kit according to the instructions provided by the manufacturer (Bio-Rad, Hercules, CA, USA).

Quantitative real-time RT-PCR

The RNA (500 ng of each sample) was reverse transcribed into cDNA by means of the iScript cDNA Synthesis kit (Bio-Rad, Mississauga, ON, Canada). The conditions for the preparation of the cDNA templates for PCR analysis were 5 min at 25°C, 1 h at 42°C, and 5 min at 85°C. Quantitative PCR (qPCR) was carried out as previously described [36]. The quantity of mRNA transcripts was measured with the Bio-Rad CFX96 real-time PCR detection system. Reactions were performed using a PCR supermix, also from Bio-Rad (iQ SYBR Green supermix). Primers (Table 6) were added to the reaction mix to a final concentration of 250 nM. Five microliters of each cDNA sample were added to a 20 μl PCR mixture containing 12.5 μl of the iQ SYBR Green supermix, 0.5 μl of specific primers ACT1, SAP2, SAP4, SAP5, SAP6, HWP1, and EAP1 (Midland Certified Reagent Company, Inc., Midland, TX, USA), as well as EFG1 and NRG1 (Invitrogen Life Technologies Inc., Burlington, ON, Canada), and 7 μl of RNase/DNase-free water (MP Biomedicals, Solon, OH, USA). Each reaction was performed in a Bio-Rad MyCycler Thermal Cycler. For the qPCR, the CT was automatically determined using the accompanying Bio-Rad CFX Manager. The thermocycling conditions for the ACT1, SAPs 2-4-5-6, and EAP1 were established as 5 min at 95°C, followed by 30 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, with each reaction performed in triplicate. For the EFG1 and NRG1, the thermocycling conditions were set for 3 min at 95°C, followed by 45 cycles of 10 s at 95°C, 40 s at 54°C, and 40 s at 72°C, with each reaction also performed in triplicate. For the HWP1, the conditions were 3 min at 95°C, followed by 45 cycles of 10 s at 95°C, 30 s at 54°C, and 40 s at 72°C, with each reaction performed in triplicate. The specificity of each primer pair was determined by the presence of a single melting temperature peak. The ACT1 produced uniform expression levels varying by less than 0.5 CTs between sample conditions and thus became the reference gene for this study. The results were analyzed by means of the $2^{-\Delta\Delta Ct}$ (Livak) relative expression method.

Table 6 Primer sequences used for the qRT-PCR

Gene	Primer sequence 5′ to 3′	Amp size (bp)
ACT1	Forward : GCTGGTAGAGACTTGACCAACCA	87
	Reverse : GACAATTTCTCTTTCAGCACTAGTAGTGA	
SAP2	Forward : TCCTGATGTTAATGTTGATTGTCAAG	82
	Reverse : TGGATCATATGTCCCCTTTTGTT	
SAP4	Forward : AGATATTGAGCCCACAGAAATTCC	82
	Reverse : CAATTTAACTGCAACAGGTCCTCTT	
SAP5	Forward : CAGAATTTCCCGTCGATGAGA	78
	Reverse : CATTGTGCAAAGTAACTGCAACAG	
SAP6	Forward : TTACGCAAAAGGTAACTTGTATCAAGA	102
	Reverse : CCTTTATGAGCACTAGTAGACCAAACG	
ALS3	Forward : AATGGTCCTTATGAATCACCATCTACTA	51
	Reverse : GAGTTTTCATCCATACTTGATTTCACAT	
HWP1	Forward : GCTCAACTTATTGCTATCGCTTATTACA	67
	Reverse : GACCGTCTACCTGTGGGACAGT	
EAP1	Forward : CTGCTCACTCAACTTCAATTGTCG	51
	Reverse : GAACACATCCACCTTCGGGA	
EFG1	Forward : TATGCCCCAGCAAACAACTG	202
	Reverse : TTGTTGTCCTGCTGTCTGTC	
NRG1	Forward : CACCTCACTTGCAACCCC	198
	Reverse : GCCCTGGAGATGGTCTGA	

Effect of KSL-W on *C. albicans* biofilm formation

C. albicans biofilms were obtained by culturing the yeast on a porous collagen scaffold which facilitated *C. albicans* penetration through the pores and its adhesion to the scaffold through collagen affinity. This also promoted biofilm formation and handling with no cell loss, thus contributing to maintaining the biofilm structure. For this purpose, 5 mm × 5 mm samples of porous scaffold (Collatape, Zimmer Dental Inc., Carlsbad, CA, USA) were placed into a 24-well plate. The scaffolds were then rinsed twice with culture medium, seeded with *C. albicans* (10^5 cells), and incubated for 30 min at 30°C without shaking to allow for adherence. Fresh Sabouraud medium was added to each well in the presence or absence of various concentrations of KSL-W (1, 10, 25, 50, 75, and 100 μg/ml). Two controls were included in this study: the negative control was *C. albicans* seeded without KSL-W, while the positive control was *C. albicans* seeded with amphotericin B (1, 5, and 10 μg/ml). The *C. albicans*-seeded scaffolds were then incubated for 2, 4, and 6 days at 30°C. The medium, KSL-W, and amphotericin B were refreshed every 48 h. Following each culture period, *C. albicans* growth and biofilm formation was assessed by scanning electron microscopy and XTT-menadione assay.

Scanning electron microscopy (SEM) analysis

Biofilms were fixed in ethylene glycol for 60 min and rinsed once with sterile PBS. Dehydration was performed in a series of 5-min treatments with ethanol

solutions of increasing concentration (50, 70, 90, and twice at 100%). The dehydrated biofilms were kept overnight in a vacuum oven at 25°C, after which time they were sputter-coated with gold, examined, and imaged (n = 4) under a JEOL 6360 LV SEM (Soquelec, Montréal, QC, Canada) operating at a 30 kV accelerating voltage.

XTT reduction assay

To support the hypothesis that KSL-W quantitatively affects *C. albicans* biofilms, an XTT reduction assay was performed on the KSL-W-treated and control biofilms at defined time points. XTT assay is one of the most useful and accurate methods to investigate microbial biofilm formation. The metabolic activity of the biofilm cells was measured as a reflection of viable cell count. To do so, *C. albicans* biofilms formed in the porous scaffold with or without KSL-W treatments for 2, 4, and 6 days were subjected to an XTT assay. Fifty microliters of XTT salt solution (1 mg/ml in PBS; Sigma-Aldrich) and 4 μl of menadione solution (1 mM in acetone; Sigma-Aldrich) were added to wells containing 4 ml of sterile PBS. The biofilms were then added to the mixture and the plates were incubated at 37°C for 5 h, after which time the supernatant was collected to measure the XTT formazan at 492 nm by means of an xMark microplate spectrophotometer (Bio-Rad, Mississauga, ON, Canada).

Effect of KSL-W on the disruption of mature *C. albicans* biofilms

Mature *C. albicans* biofilms were obtained by culturing *C. albicans* (10^5) on a porous 3D collagen scaffold for 6 days at 30°C in Sabouraud liquid medium supplemented with 0.1% glucose at pH 5.6. The culture medium was refreshed every 2 days. At the end of the 6-day culture period, the biofilms were treated (or not) with KSL-W (75 and 100 μg/ml). Amphotericin B-treated biofilms (1, 5, and 10 μg/ml) were used as the positive controls. The biofilms were continuously incubated (or not) with either KSL-W or amphotericin B for 2, 4, and 6 days, with medium changing every day. KSL-W and amphotericin B were also refreshed at each medium changing. Following each incubation period, SEM and XTT analyses were performed, as described above.

Statistical analysis

Each experiment was performed at least four times, with experimental values expressed as means ± SD. The statistical significance of the differences between the control (absence of KSL-W) and test (presence of KSL-W or amphotericin B) values was determined by means of a one-way ANOVA. Posteriori comparisons were performed using Tukey's method. Normality and variance assumptions were verified using the Shapiro-Wilk test and the Brown and Forsythe test, respectively. All of the assumptions were fulfilled. P values were declared significant at ≤ 0.05. The data were analyzed using the SAS version 8.2 statistical package (SAS Institute Inc., Cary, NC, USA).

Authors' contributions

MR, KPL, and AS designed the experiments, supervised the research and wrote the paper. ST, AA, and AS performed the experiments and data analyses and contributed to the writing of the paper. Each author read and approved the final manuscript.

Acknowledgements

This study was supported financially by the United States Army Medical Research and Materiel Command (Award number ERMS No. 12304006) and by a grant from the Fonds Émile-Beaulieu, a Université Laval foundation. The authors also thank Ms. Claire Kingston (Traduction CFK) for proofreading and editing this manuscript.

DOD Disclaimer

One of the authors (KPL) is a United States Government employee. The work presented is part of his official duties. The opinions or assertions contained herein are the personal views of these authors and are not to be construed as official or reflecting the views of the United States Army or Department of Defense.

Author details

[1]Oral Ecology Research Group, Faculty of Dentistry, Laval University, 2420, rue de la Terrasse, Quebec G1V 0A6, QC, Canada. [2]Department of Biochemistry, Genome Research Chair, College of Science King Saud University, Riyadh, Kingdom of Saudi Arabia. [3]Dental and Trauma Research Detachment, US Army Institute of Surgical Research, JBSA Fort Sam Houston, San Antonio, TX, USA.

Received: 5 July 2013 Accepted: 4 November 2013
Published: 7 November 2013

References

1. Arendorf TM, Walker DM: **The prevalence and intra-oral distribution of Candida albicans in man.** *Arch Oral Biol* 1980, **25:**1–10.
2. Cannon RD, Chaffin WL: **Oral colonization by Candida albicans.** *Crit Rev Oral Biol Med* 1999, **10:**359–383.
3. Sudbery P, Gow N, Berman J: **The distinct morphogenic states of *Candida albicans*.** *Trends Microbiol* 2004, **12:**317–324.
4. Nobile CJ, Nett JE, Andes DR, Mitchell AP: **Function of *Candida albicans* adhesin Hwp1 in biofilm formation.** *Eukaryot Cell* 2006, **5:**1604–1610.
5. Li F, Palecek SP: ***EAP1*, a *Candida albicans* gene involved in binding human epithelial cells.** *Eukaryot Cell* 2003, **2:**1266–1273.
6. Sohn K, Urban C, Brunner H, Rupp S: ***EFG1* is a major regulator of cell wall dynamics in *Candida albicans* as revealed by DNA microarrays.** *Mol Microbiol* 2003, **47:**89–102.
7. Stoldt VR, Sonneborn A, Leuker CE, Ernst JF: **Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi.** *EMBO J* 1997, **16:**1982–1991.
8. Lo HJ, Köhler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR: **Nonfilamentous C. albicans mutants are avirulent.** *Cell* 1997, **90:**939–949.
9. Schaller M, Borelli C, Korting HC, Hube B: **Hydrolytic enzymes as virulence factors of *Candida albicans*.** *Mycoses* 2005, **48:**365–377.
10. Décanis N, Tazi N, Correia A, Vilanova M, Rouabhia M: **Farnesol, a fungal quorum-sensing molecule triggers Candida albicans morphological changes by down-regulating the expression of different secreted aspartyl proteinase genes.** *Open Microbiol J* 2011, **5:**119–126.
11. Naglik JR, Challacombe SJ, Hube B: ***Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis.** *Microbiol Mol Biol Rev* 2003, **67:**400–428.
12. Hube B, Naglik J: ***Candida albicans* proteinases: resolving the mystery of a gene family.** *Microbiology* 2001, **147:**1997–2005.

13. White TC, Miyasaki SH, Agabian N: **Three distinct secreted aspartyl proteinases in *Candida albicans*.** *J Bacteriol* 1993, **175:**6126–6133.
14. White TC, Agabian N: ***Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors.** *J Bacteriol* 1995, **177:**5215–5221.
15. Albrecht A, Felk A, Pichova I, Naglik JR, Schaller M, de Groot P, Maccallum D, Odds FC, Schäfer W, Klis F, Monod M, Hube B: **Glycosylphosphatidylinositol-anchored proteases of Candida albicans target proteins necessary for both cellular processes and host-pathogen interactions.** *J Biol Chem* 2006, **281**(2):688–694.
16. van der Weerden NL, Bleackley MR, Anderson MA: **Properties and mechanisms of action of naturally occurring antifungal peptides.** *Cell Mol Life Sci* 2013, **70**(19):3545–3570.
17. Fjell CD, Hiss JA, Hancock RE, Schneider G: **Designing antimicrobial peptides: form follows function.** *Nat Rev Drug Discov* 2012, **11:**37–51.
18. Seo MD, Won HS, Kim JH, Mishig-Ochir T, Lee BJ: **Antimicrobial peptides for therapeutic applications: a review.** *Molecules* 2012, **17:**12276–12286.
19. Campbell EL, Serhan CN, Colgan SP: **Antimicrobial aspects of inflammatory resolution in the mucosa: a role for proresolving mediators.** *J Immunol* 2011, **187:**3475–3481.
20. Lehrer RI, Lu W: **alpha-Defensins in human innate immunity.** *Immunol Rev* 2012, **245:**84–112.
21. Mehra T, Koberle M, Braunsdorf C, Mailander-Sanchez D, Borelli C, *et al*: **Alternative approaches to antifungal therapies.** *Exp Dermatol* 2012, **21:**778–782.
22. Zhu S: **Discovery of six families of fungal defensin-like peptides provides insights into origin and evolution of the CSalphabeta defensins.** *Mol Immunol* 2008, **45:**828–838.
23. Batoni G, Maisetta G, Brancatisano FL, Esin S, Campa M: **Use of antimicrobial peptides against microbial biofilms: advantages and limits.** *Curr Med Chem* 2011, **18:**256–279.
24. Dziarski R, Gupta D: **Review: Mammalian peptidoglycan recognition proteins (PGRPs) in innate immunity.** *Innate Immun* 2010, **16:**168–174.
25. Taraszkiewicz A, Fila G, Grinholc M, Nakonieczna J: **Innovative strategies to overcome biofilm resistance.** *Biomed Res Int* 2013, **2013:**150653. doi: 10.1155/2013/150653.
26. Cota-Arriola O, Cortez-Rocha MO, Burgos-Hernandez A, Ezquerra-Brauer JM, Plascencia-Jatomea M: **Controlled release matrices and micro/nanoparticles of chitosan with antimicrobial potential: development of new strategies for microbial control in agriculture.** *J Sci Food Agric* 2013, **93:**1525–1536.
27. Dhople V, Krukemeyer A, Ramamoorthy A: **The human beta-defensin-3, an antibacterial peptide with multiple biological functions.** *Biochim Biophys Acta* 2006, **1758:**1499–1512.
28. Joly S, Maze C, McCray PB Jr, Guthmiller JM: **Human beta-defensins 2 and 3 demonstrate strain-selective activity against oral microorganisms.** *J Clin Microbiol* 2004, **42:**1024–1029.
29. Mooney C, Haslam NJ, Pollastri G, Shields DC: **Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity.** *PLoS One* 2012, **7:**e45012.
30. Na DH, Faraj J, Capan Y, Leung KP, DeLuca PP: **Stability of antimicrobial decapeptide (KSL) and its analogues for delivery in the oral cavity.** *Pharm Res* 2007, **24:**1544–1550.
31. Hong SY, Park TG, Lee KH: **The effect of charge increase on the specificity and activity of a short antimicrobial peptide.** *Peptides* 2001, **22:**1669–1674.
32. Oh JE, Hong SY, Lee KH: **Structure-activity relationship study: short antimicrobial peptides.** *J Pept Res* 1999, **53:**41–46.
33. Concannon SP, Crowe TD, Abercrombie JJ, Molina CM, Hou P, *et al*: **Susceptibility of oral bacteria to an antimicrobial decapeptide.** *J Med Microbiol* 2003, **52:**1083–1093.
34. Leung KP, Crowe TD, Abercrombie JJ, Molina CM, Bradshaw CJ, *et al*: **Control of oral biofilm formation by an antimicrobial decapeptide.** *J Dent Res* 2005, **84:**1172–1177.
35. Baker PJ, Coburn RA, Genco RJ, Evans RT: **The in vitro inhibition of microbial growth and plaque formation by surfactant drugs.** *J Periodontal Res* 1978, **13:**474–485.
36. Semlali A, Leung KP, Curt S, Rouabhia M: **Antimicrobial decapeptide KSL-W attenuates Candida albicans virulence by modulating its effects on Toll-like receptor, human β-defensin, and cytokine expression by engineered human oral mucosa.** *Peptides* 2011, **32**(5):859–867.
37. Okkers DJ, Dicks LM, Silvester M, Joubert JJ, Odendaal HJ: **Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from Lactobacillus pentosus with a fungistatic effect on Candida albicans.** *J Appl Microbiol* 1999, **87:**726–734.
38. Dixon DR, Jeffrey NR, Dubey VS, Leung KP: **Antimicrobial peptide inhibition of Porphyromonas gingivalis 381-induced hemagglutination is improved with a synthetic decapeptide.** *Peptides* 2009, **30:**2161–2167.
39. Raines SM, Rane HS, Bernardo SM, Binder JL, Lee SA, *et al*: **Deletion of Vacuolar Proton-translocating ATPase Voa Isoforms Clarifies the Role of Vacuolar pH as a Determinant of Virulence-associated Traits in Candida albicans.** *J Biol Chem* 2013, **288:**6190–6201.
40. Ariyachet C, Solis NV, Liu Y, Prasadarao NV, Filler SG, *et al*: **SR-Like RNA-Binding Protein Slr1 Affects Candida albicans Filamentation and Virulence.** *Infect Immun* 2013, **81:**1267–1276.
41. Décanis N, Savignac K, Rouabhia M: **Farnesol promotes epithelial cell defense against Candida albicans through Toll-like receptor 2 expression, interleukin-6 and human beta-defensin 2 production.** *Cytokine* 2009, **45:**132–140.
42. Zhang J, Silao FG, Bigol UG, Bungay AA, Nicolas MG, *et al*: **Calcineurin is required for pseudohyphal growth, virulence, and drug resistance in Candida lusitaniae.** *PLoS One* 2012, **7:**e44192.
43. Koshlukova SE, Araujo MWB, Baev D, Edgerton M: **Released ATP is an extracellular cytotoxic mediator in salivary histatin 5-induced killing of*Candida albicans*.** *Infect Immun* 2000, **68:**6848–6856.
44. Vylkova S, Jang WS, Li W, Nayyar N, Edgerton M: **Histatin 5 initiates osmotic stress response in *Candida albicans* via activation of the Hog1 mitogen-activated protein kinase pathway.** *Eukaryot Cell* 2007, **6:**1876–1888.
45. Jang WS, Bajwa JS, Sun JN, Edgerton M: **Salivary histatin 5 internalization by translocation, but not endocytosis, is required for fungicidal activity in *Candida albicans*.** *Mol Microbiol* 2010, **77:**354–370.
46. Ramage G, Vandewalle K, Wickes BL, Lopez-Ribot JL: **Characteristics of biofilm formation by Candida albicans.** *Rev Iberoam Micol* 2001, **18:**163–170.
47. Banerjee M, Uppuluri P, Zhao XR, Carlisle PL, Vipulanandan G, *et al*: **Expression of UME6, a key regulator of Candida albicans hyphal development, enhances biofilm formation via Hgc1- and Sun41-dependent mechanisms.** *Eukaryot Cell* 2013, **12:**224–232.
48. da Silva BR, de Freitas VA, Carneiro VA, Arruda FV, Lorenzon EN, *et al*: **Antimicrobial activity of the synthetic peptide Lys-a1 against oral streptococci.** *Peptides* 2013, **42C:**78–83.
49. Beckloff N, Laube D, Castro T, Furgang D, Park S, *et al*: **Activity of an antimicrobial peptide mimetic against planktonic and biofilm cultures of oral pathogens.** *Antimicrob Agents Chemother* 2007, **51:**4125–4132.
50. Patrzykat A, Friedrich CL, Zhang L, Mendoza V, Hancock RE: **Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in Escherichia coli.** *Antimicrob Agents Chemother* 2002, **46:**605–614.
51. Mason AJ, Chotimah IN, Bertani P, Bechinger B: **A spectroscopic study of the membrane interaction of the antimicrobial peptide Pleurocidin.** *Mol Membr Biol* 2006, **23:**185–194.
52. Bauerova V, Pichova I, Hruskova-Heidingsfeldova O: **Nitrogen source and growth stage of Candida albicans influence expression level of vacuolar aspartic protease Apr1p and carboxypeptidase Cpy1p.** *Can J Microbiol* 2012, **58:**678–681.
53. Cleary IA, Lazzell AL, Monteagudo C, Thomas DP, Saville SP: **BRG1 and NRG1 form a novel feedback circuit regulating Candida albicans hypha formation and virulence.** *Mol Microbiol* 2012, **85:**557–573.
54. Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, *et al*: **A recently evolved transcriptional network controls biofilm development in Candida albicans.** *Cell* 2012, **148:**126–138.
55. Murad AM, Leng P, Straffon M, Wishart J, Macaskill S, *et al*: **NRG1 represses yeast-hypha morphogenesis and hypha-specific gene expression in Candida albicans.** *EMBO J* 2001, **20:**4742–4752.
56. Braun BR, Kadosh D, Johnson AD: **NRG1, a repressor of filamentous growth in C.albicans, is down-regulated during filament induction.** *EMBO J* 2001, **20:**4753–4761.
57. Li F, Svarovsky MJ, Karlsson AJ, Wagner JP, Marchillo K, *et al*: **Eap1p, an adhesin that mediates Candida albicans biofilm formation in vitro and in vivo.** *Eukaryot Cell* 2007, **6:**931–939.
58. Sharkey LL, McNemar MD, Saporito-Irwin SM, Sypherd PS, Fonzi WA: **HWP1 functions in the morphological development of Candida albicans down-stream of EFG1, TUP1, and RBF1.** *J Bacteriol* 1999, **181:**5273–5279.
59. Staniszewska M, Bondaryk M, Siennicka K, Kurek A, Orlowski J, *et al*: **In vitro study of secreted aspartyl proteinases Sap1 to Sap3 and Sap4 to Sap6

expression in Candida albicans pleomorphic forms. *Pol J Microbiol* 2012, **61**:247–256.
60. Lian CH, Liu WD: **Differential expression of Candida albicans secreted aspartyl proteinase in human vulvovaginal candidiasis.** *Mycoses* 2007, **50**:383–390.
61. Hube B, Monod M, Schofield DA, Brown AJ, Gow NA: **Expression of seven members of the gene family encoding secretory aspartyl proteinases in Candida albicans.** *Mol Microbiol* 1994, **14**:87–99.
62. Puri S, Kumar R, Chadha S, Tati S, Conti HR, *et al*: **Secreted aspartic protease cleavage of Candida albicans Msb2 activates Cek1 MAPK signaling affecting biofilm formation and oropharyngeal candidiasis.** *PLoS One* 2012, **7**:e46020.
63. Hong SY, Oh JE, Kwon M, Choi MJ, Lee JH, *et al*: **Identification and characterization of novel antimicrobial decapeptides generated by combinatorial chemistry.** *Antimicrob Agents Chemother* 1998, **42**:2534–2541.
64. Denizot F, Lang R: **Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability.** *J Immunol Methods* 1986, **89**:271–277.
65. Li L, Zhang C, Konopka JB: **A *Candida albicans* temperature-sensitive cdc12-6 mutant identifies roles for septins in selection of sites of germ tube formation and hyphal morphogenesis.** *Eukaryot Cell* 2012, **11**:1210–1218.

doi:10.1186/1471-2180-13-246
Cite this article as: Theberge *et al.*: *C. albicans* growth, transition, biofilm formation, and gene expression modulation by antimicrobial decapeptide KSL-W. *BMC Microbiology* 2013 **13**:246.

Appendix 1

Le décapeptide KSL-W réduit la croissance de *Candida albicans* et dégrade les biofilms en diminuant l'expression de plusieurs gènes de virulence.

Theberge Simon[1], Semlali Abdelhabib[1,2], Alamri Abdullah[1], Leung P. Kai[3], and Rouabhia Mahmoud[1]

[1]Oral Ecology Research Group, Faculty of Dentistry, Laval University, Quebec, QC, Canada,
[2]Genome Research Chair, Department of Biochemistry, College of Science King Saud University, Riyadh, Kingdom of Saudi Arabia
[3]Dental and Trauma Research Detachment, US Army Institute of Surgical Research, Fort Sam Houston, TX, United States

Introduction : *Candida albicans* est le plus fréquent pathogène fongique impliqué dans les infections nosocomiales en Amérique du Nord[1]. Suivant l'utilisation courante d'antifongiques, *C. albicans* peut développer des résistances aux traitements conventionnels. Afin de pallier cet obstacle, le décapeptide KSL-W a été développé et présente un large spectre antimicrobien pouvant affecter *C. albicans*[2,3].
L'objectif de cette étude est d'évaluer l'effet du KSL-W sur la croissance, la transformation de *C. albicans*, ainsi que sur l'expression des gènes impliqués dans la virulence de *C. albicans*.
Matériels et Méthodes : L'effet du KSL-W a été étudié en analysant la transformation de *C. albicans-SC5314* en présence et en absence de KSL-W à l'aide de suivis microscopiques. Des analyses spécifiques au MTT et au XTT ont été réalisées afin de déterminer l'effet du KSL-W sur la prolifération ainsi que sur la formation et la dégradation de biofilm. Ces travaux ont été soutenus par l'analyse de l'expression des gènes *Sap2*, *Sap4*, *Sap5*, *Sap6*, *HWP1*, *EAP1*, *EFG1* et *NRG1* par la technique RT-qPCR. Tous les effets ont été comparés à l'amphotéricine B, un antifongique utilisé en clinique.
Résultats et conclusions : La transformation levure-hyphe est inhibée à partir de concentrations de 5μg/ml. La prolifération est diminuée suivant une exposition de 5h à des concentrations de 10μg/ml et persiste jusqu'à 10h avec des concentrations ≥50μg/ml. La formation de biofilm est inhibée par des concentrations de ≥25μg/ml. Les biofilms sont dégradés à partir de concentrations de 75μg/ml. Le KSL-W réprime l'expression des gènes de virulence *Sap2*, *Sap4*, *Sap5*, *Sap6*, *HWP1*, *EAP1*. Les gènes *EFG1* et *NRG1* ont été régulés à la hausse par le KSL-W. Les effets sont comparables à l'amphotéricine B. Par ses effets sur les facteurs de virulence et par son effet sur les gènes de virulence, le KSL-W présente une alternative de traitement intéressante dans le contrôle des infections fongique à *C. albicans* (This study was supported financially by the United States Army Medical Research and Materiel Command (Award number ERMS No. 12304006) and by a grant from the Fonds Émile-Beaulieu, a Université Laval foundation).

Références :

1. Wisplinghoff, H. et al. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **39**, 309–17 (2004).
2. Semlali, A. *et al.* Antimicrobial decapeptide KSL-W attenuates Candida albicans virulence by modulating its effects on Toll-like receptor, human β-defensin, and cytokine expression by engineered human oral mucosa. *Peptides* **32**, 859–67 (2011).
3. Leung, K.-P. *et al.* Control of Oral Biofilm Formation by an Antimicrobial Decapeptide. *Journal of Dental Research* **84**, 1172–1177 (2005).

Appendix 2 :

Journée de la recherche faculté de médecine – 30 mai – soumission avant 4 avril

Un nouveau peptide antimicrobien contrôle la virulence de Candida en réduisant sa viabilité via un processus d'apoptose et de nécrose.

THEBERGE SIMON[1], Jacques Éric[1] and Leung Kai P[2] and Rouabhia Mahmoud[1]

[1] Groupe de recherche en écologie buccale, Université Laval
[2] Microbiology Branch, US Army Dental and Trauma Research Detachment, Institute of Surgical Research, Fort Sam Houston, TX, USA

OBJECTIF : Le décapeptide KSL-W présente un large spectre antimicrobien affectant plusieurs pathogènes dont *S. mutans* et *C albicans*. Ce peptide semble réduire la croissance de C. albicans ainsi que la formation de biofilm en agissant sur certains gènes spécifiques. Cependant les mécanismes d'action du KSL-W ne sont pas encore élucidés. Dans cette étude, nous avons évalué la cinétique et l'atteinte de l'intégrité morphologique ainsi que le type de mort cellulaire (apoptotique/nécrotique) induites par le KSL-W sur C. albicans en comparaison à une autre souche soit : *C. parapsilosis*.
MÉTHODES : Les souches de Candida ont été mises en culture en présence et en absence de KSL-W. l'effet de KSL-W sur la viabilité cellulaire a été déterminé à l'aide du test d'exclusion du bleu trypan. Ces travaux ont été confirmés par des analyses de l'activité apoptotique ou nécrotique du KSL-W par cytométrie en flux à l'aide d'un marquage à l'annexin V-FITC/PI. Des analyses par microscopie électronique à transmission ont été effectuées afin de visualiser l'effet sur la paroi cellulaire et les autres composantes intracellulaires. Les effets du KSL-W ont tous été comparés à ceux de l'amphotéricine B.
RÉSULTATS : L'effet antifongique du KSL-W s'amorce dès les premières 30 minutes d'exposition de *C. albicans* et *C. parapsilosis* au KSL-W à des doses de 10 µg/ml. Le KSL-W induit également la nécrose de *C. albicans* à partir de concentrations de 1µg ml-1 (49,0%) et 25µg/ml (97,7%). L'activité du KSL-W sur *C parapsilosis* est principalement apoptotique (42,2%) après 3 heures d'exposition à 25µg/ml de KSL-W. Les analyses en microscopie à transmission montrent une atteinte de la membrane de *C. albicans* et de *C. parapsilosis* suivant des traitements de KSL-W à 25µg/ml.
CONCLUSIONS : Nos travaux démontrent une activité antifongique de KSL-W. Cette activité est parfois comparable, parfois non comparable à celle de l'amphotéricine B, (suggérant un mécanisme d'action différent de celui de l'amphotéricine B). Ces travaux suggèrent l'utilisation du KSL-W comme molécule de choix pour le contrôle des infections fongiques à *Candida.*

Appendix 3

Titre : Le KSL-W réduite la croissance Candida albicans et la formation de biofilm en diminuant l'expression de plusieurs gène de virulence.

Auteurs : Simon Théberge, Abdelhabib Semlali, Kai P Lung, Abdullah Alamri and Mahmoud Rouabhia

Présentation : Poster

Objectifs : Le décapeptide α-hélicoïdal synthétique, le KSL-W (KKVVFWVKFK) possède un large spectre affectant plusieurs souches de pathogènes bactériens oraux. Cependant effet de ce peptide sur des souches fongiques dont Candida *albicans* reste à démontrer. Le but est d'étudier de l'effet du KSL-W sur les différents facteurs de virulence du C. *albicans*. **Méthodologie :** L'effet de KSL-W a été étudié en analysant la transformation, la prolifération en utilisant la microscopie et le MTT. Ces travaux ont été supportés par des analyses spécifiques à la formation de biofilm (XTT) et à l'activation de certains gènes à l'aide de la technique qRT-PCR. Une analyse de l'activité apoptotique/anti-apoptotique du KSL-W a été réalisée à l'aide d'Annexin V-FITC/IP. Tous les effets étudiés du KSL-W ont été comparés à l'amphotéricine B. **Résultats :** Le KSL-W inhibe la transformation à partir de concentrations de 5µg ml^{-1}. Il s'est montré efficace à inhiber la formation de biofilms à des concentrations de 50µg ml^{-1} et à réduire la viabilité à l'intérieur d'un biofilm mature à des concentrations de 50µg ml^{-1} et est d'efficacité comparable à l'amphotéricine B dans les deux cas. Les analyses ultrastucturales confirment l'efficacité du KSL-W à réduire et à dégrader le biofilm.. L'efficacité du KSL-W passe aussi par la réduction de l'expression de plusieurs gènes dont SAP2, 4, 5, 6, EFG1 et HWP1 impliqués dans la pathogénèse de C. albicans. . **Conclusion : Par ses effets importants sur C. albicans,** le KSL-W pourrait être considéré dans le contrôle de Candida *albicans* ((This study was supported financially by the United States Army Medical Research and Materiel Command (Award number ERMS No. 12304006) and by a grant from the Fonds Émile-Beaulieu, a Université Laval foundation).